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ABSTRACT

Hydrogen peroxide is an unavoidable by-product of cell metabolism, but when it is not properly managed by the body it can lead to several pathologies (e.g., premature aging, cardiovascular and neurodegenerative diseases, cancer). Several methods have been proposed for the measurement of intracellular H_2O_2 but none of them has proven to be selective. We developed a rapid all-in-one chemiluminescent bioassay for the quantification of H_2O_2 in living cells with a low limit of detection (0.15 μM). The method relies on an adamantylidene-1,2-dioxetane lipophilic probe containing an arylboronate moiety; upon reaction with H_2O_2 the arylboronate moiety is converted to the correspondent phenol and the molecule decomposes leading to an excited-state fragment that emits light. The probe has been successfully employed for quantifying intracellular H_2O_2 in living human endothelial, colon and keratinocyte cells exposed to different pro-oxidant stimuli (i.e., menadione, phorbol myristate acetate and lipopolysaccharide). Imaging experiments clearly localize the chemiluminescence emission inside the cells. Treatment of cells with antioxidant molecules leads to a dose-dependent decrease of intracellular H_2O_2 levels. As a proof of concept, the bioassay has been used to measure the antioxidant activity of extracts from Brassica juncea wastes, which contain glucosinolates, isothiocyanates and other antioxidant molecules.

1. Introduction

In recent years, free radicals have gained increasing importance in biomedicine, owing to their dual role in the human body. Emerging data indicate that generation and reactivity of free radicals and other oxidants are harnessed to regulate numerous redox-dependent physiological processes. In turn, uncontrolled production and deregulation of redox signalling are implicated in the initiation and propagation of several pathological conditions, such as cardiometabolic, neurodegenerative and cancer diseases [1]. Since reactive oxygen and nitrogen species include a broad range of chemically distinct species with diverse biological reactivities, it is essential to detect, localize and characterize these species accurately in biological systems to clearly attribute a particular cell event to a specific chemical entity.

Hydrogen peroxide (H_2O_2) is one of the major members of reactive oxygen species (ROS) in living organisms and plays an important role in the regulation of a variety of biological processes [2].

However, when it is not properly managed by the body or is produced in excess (as under certain kinds of stress) it can lead to chronic pathologies [3]. In addition, various ROS are converted to H_2O_2 within cells [4], therefore a change in H_2O_2 level can reasonably reflect a general change in the intracellular ROS production.

Several bioanalytical approaches have been proposed to detect intracellular H_2O_2 [5]. The most common technique consists in the use of fluorescent probes such as 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), Amplex Red, dihydrorhodamine, phosphine-based fluorophores, lanthanide coordination complexes, and redox-responsive fluorescent substances [6,7]. However, current fluorescent probes have several limitations, the main one being their insufficient selectivity to H_2O_2 over other ROS, which is critical for the accurate measurement of

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intracellular H_2O_2 levels [8,9]. For example, the fluorescent probe $DCFH_2$-DA, widely used to detect intracellular oxidant species, does not directly react with H_2O_2 and its fluorescence results from the interaction with strong oxidants, such as those produced from metal ion- and peroxidase-catalyzed reactions of H_2O_2 and from the decomposition of $ONOO^-$ [8,10]. Indeed, we found that the fluorescence signal of $DCFH_2$ was enhanced by the concentration of Fe^{2+}, while for a fixed amount of Fe^{2+} no correlation was observed between the fluorescence emission and the concentration of H_2O_2 up to 100 μM [11]. In addition, tissues, individual cells and subcellular structures scatter visible light extensively [12], thus scattering of fluorescence emission occurs at the surface and inside live cells. Consequently, the use of fluorescent probes in imaging of biological samples may suffer from low signal-to-noise due to scattering of emitted fluorescence and sample autofluorescence. Poor photostability due to photobleaching could also represent an issue for some fluorophores [8].

Therefore, novel selective probes with reduced background interferences are required to perform quantitative studies on intracellular H_2O_2.

Chemiluminescence (CL)-based methods are gaining increased interest for studying biological systems, as they maintain the simplicity of optical techniques and provide easy signal quantification and high signal-to-noise ratios [13]. The absence of any external excitation light source is particularly attractive because it avoids the drawbacks of fluorescent probes, also facilitating imaging of relatively thick specimens as inferred from recent animal studies [14,15]. On the other hand, the main limitation of CL is the weakness of the light signal and possible interference in the chemistry of the CL reaction (pH, ionic strength, etc). Chemiluminescence also offers some advantages over bioluminescence (BL) which could be use alternatively, as light generation can be initiated by a specific chemical reaction without further enzymatic dependency and no genetic cell engineering is required to express BL enzymes such as luciferases [16].

Spontaneous ultraweak photon emission, which is related to by-products of the chemical reactions of cell metabolisms, has been used to monitor ROS in cells, However, its applicability in biomedical studies is limited by the very low signal intensity that requires the use of ultra-high sensitive photon counting systems [17]. Chemiluminescent probes such as luminol, lucigenin and their derivatives, whose oxidation gives reasonably high yields of electronically excited, light-emitting products (the CL quantum yields of luminol and lucigenin are 0.012 [18] and 0.01–0.02 [19], respectively) solved this issue by increasing the photon output by several orders of magnitude. These probes have been applied in cell-based assays to investigate respiratory burst in polymorphonuclear neutrophils and phagocytes and to non-invasively image ROS and reactive nitrogen species (RNS) in living mice under pro-inflammatory conditions [20,21]. However, these compounds have low bioavailability and cannot discriminate between different ROS species. So far, only a few CL systems specific for the selective detection of H_2O_2 and suitable for quantitative assays or *in vitro* and *in vivo* imaging have been described. For example, nanoparticles containing a peroxalate derivative and a fluorescent acceptor have been employed for H_2O_2 measurements, although reports regard *in vivo* imaging in mice models [22–24] or *in vitro* measurements in cells challenged with the exogenous addition of H_2O_2 [25] rather than evaluation of increase of intracellular H_2O_2 as a result of physiological pro-oxidant stimuli (such as inflammatory cytokines).

Recently, a new family of CL dioxetane-based luminophores that emit light under physiological conditions (i.e. pH 7.4) with high efficiency has been developed [26]. Since spontaneous decomposition and chemiexcitation followed by light emission of such molecules can be triggered by the presence of a phenolic group, specific turn-on CL probes have been obtained by introducing in the luminophore a phenolic function masked by target-responsive groups. Chemiluminescent probes based on these luminophores have been successfully developed for the selective detection of various species, from enzymes (e.g., alkaline phosphatase, β-galactosidase and nitroreductase) to molecules such as formaldehyde, peroxynitrite and H_2O_2 [26]. In particular, the H_2O_2 CL probe employed an aromatic boronate as the target-responsive group. Indeed, aromatic boronates, which have already been exploited to increase the selectivity of fluorescent H_2O_2 probes, are particularly suited as responsive groups for CL dioxetane-based luminophores because they react with H_2O_2 to form the corresponding phenol with reaction yields close to 100% [27,28].

In this work, we exploited for the first time the possibility of using a dioxetane-based CL probe containing a boronate moiety selective for H_2O_2 [29,30] for quantitative monitoring of the intracellular production of H_2O_2 in different types of human living cells, i.e., endothelial, intestinal and keratinocyte cells, and evaluation of its inhibition by antioxidants. As a proof of concept, the bioassay has been used to measure the antioxidant activity of extracts from different tissues of a *Brassica juncea* "broad-leaf" selection which has a high biomass production and contains glucosinolates (GSLs), isothiocyanates (ITCs) and other antioxidant molecules characteristic of *Brassicaceae* family [31]. Our aim was to set up and validate an *in vitro* highly predictive cell-based assays capable to mimic the physiological oxidative stress and its response to external stimuli without employing any genetic manipulation.

2. Materials and methods

2.1. Chemicals

The dioxetane CL probe (AquaSpark™ Peroxide Probe) was kindly provided by Biosynth (Staad, Switzerland). A 10 mM stock solution was prepared by dissolving the probe in DMSO (the CL probe solution is stable for months when stored at 4 °C and protected from light).

Phosphate-buffered saline (PBS) tabs (giving a 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4), H_2O_2 solution, gelatine from bovine skin, trypsin-EDTA, oxidative stress inductors (menadione, phorbol myristate acetate - PMA and lipopolysaccharide - LPS), antioxidants (Trolox, lipoic acid and gallic acid), phenethylisothiocyanate, allyl isothiocyanate and N(G)-monomethyl-L-arginine monoacetate (L-NMMA) were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were of the highest analytical grade.

Stock solutions of the pro-oxidants menadione (100 mM), phorbol myristate acetate (PMA, 10 mM) and lipopolysaccharide (LPS, 5 mg mL^{-1}) were prepared by dissolving the compounds in DMSO (menadione and PMA) or PBS (LPS). Stock solutions of antioxidants (Trolox, lipoic acid and gallic acid) were obtained by dissolving the antioxidants in DMSO to a final concentration of 10 mM.

Dulbecco's Modified Eagle Medium (DMEM) high glucose and MEM Non-Essential Amino Acids solution 100X were purchased from Microgem (Naples, Italy). M200 medium, Low Serum Growth Supplements 50X (LSGS) and Fetal Bovine Serum (FBS) were from Thermo Fischer Scientific (Waltham, MA, USA). Antibiotic solution 100X (10,000 U/mL penicillin and 10 mg mL^{-1} streptomycin) was purchased from Sigma-Aldrich.

The ROS-Glo™ H_2O_2 Assay and the Cytotoxicity LDH Assay kits were purchased from Promega (Madison, Wisconsin, USA) and Dojindo Molecular Technologies (Rockville, MD, USA), respectively.

2.2. Plant material

Brassica juncea "broad-leaf" selection was provided from the *Brassicaceae* seed collection of CREA-CI [31]. This *B. juncea* has some interesting peculiarities with respect to the other varieties, such as a high biomass production both in pot and open field. Two plants for container were grown in square pots (22 cm side and 8 L volume). Tissue samples were collected at completely developed inflorescences. Four plants randomly chosen from 6 pots were harvested, and roots,

stem, and leaves tissues were separately collected and immediately frozen. Samples were finally freeze-dried and finely powdered to 0.5 mm size. Extraction and characterization of bioactive molecules are described in supplementary materials.

2.3. Cell cultures

Human umbilical vein endothelial cells (HUVEC), purchased from Life Technologies, were plated on gelatin-coated tissue culture dishes and maintained in phenol red-free basal medium M200 containing 10% FBS and LSGS at 37 °C with 5% CO_2. Actively proliferating cells (passages 3 to 7, 70–90% confluency) were harvested for experiments. Caco-2 cells, obtained from the American Type Culture Collection (ATCC), were maintained in DMEM high glucose containing 10% FBS, 2.5 mM L-glutamine, Antibiotic-Antimycotic solution and MEM Non-Essential Amino Acids solution. HaCaT cells, kindly gifted by Prof. F. Martini (University of Ferrara), were maintained in DMEM high glucose containing 10% FBS, 2.5 mM L-glutamine and Antibiotic-Antimycotic solution.

2.4. Cytotoxicity assay

Lactate dehydrogenase (LDH) release from cells was monitored in the culture medium using a spectrophotometric LDH assay (Cytotoxicity LDH Assay Kit). The assay was performed in the standard 96-well microtiter plate format according to manufacturer's instructions and the absorbance increase after 24 h of incubation at 37 °C was monitored using a Varioskan™ Flash Multimode Reader (Thermo Scientific) [32].

2.5. Bioassay procedure

2.5.1. Principle of the bioassay

The selective CL probe for H_2O_2 designed by Shabat's research group [27] was based on the previous Schaap's adamantylidene−-dioxetane probes [33]. Adamantylidene−dioxetane probes are single-component CL systems characterized by reasonable aqueous solubility, and therefore they do not require to be included in nanoparticles. For these features, such probes are ideally suited for intracellular imaging of H_2O_2 with negligible cytotoxicity and/or cellular perturbation effects. Differently from the Schaap's probes, the presence of an electron-withdrawing acrylic group in the aromatic ring results in the production of excited species that emits light with greater efficiency in aqueous solution, thus increasing CL emission intensity. Detection of H_2O_2 relies on a deprotection mechanism (Fig. 1). Nucleophilic addition of H_2O_2 to the boronate ester **(1)** generates the charged tetrahedral boronate complex **(2)**, which undergoes a "1,2-insertion" by the C–B bond migration to one of the electrophilic peroxide oxygens. The borate ester **(3)** is then hydrolyzed to the phenol generating an unstable phenolate−dioxetane species **(4)**, which produces the excited-state benzoate ester **(5)** *via* a chemically-initiated electron-exchange luminescence (CIEEL) mechanism. The excited benzoate ester then decays to its ground state **(6)** with emission of photons at 540 nm.

2.5.2. Calibration curve for quantification of H_2O_2

The dioxetane CL probe working solution (10 μM) was prepared by diluting the 10 mM stock solution with PBS. The H_2O_2 standard solutions for generating the calibration curve (concentration range 0.5–40 μM) were prepared in PBS. For the cell-free CL measurement, 100 μL of the H_2O_2 standard solutions (PBS for the blank) were dispensed in the wells of a 96-well black microtiter plate, then the reaction was started by adding 100 μL of the dioxetane CL probe working solution and the resulting CL emission was monitored for 60 min using a luminometric plate reader (Thermo Fisher Scientific). The calibration curve was obtained by plotting the CL signal (evaluated as the CL emission integrated between 40 and 60 min after addition of the CL probe) versus the actual concentration of H_2O_2 and fitting the experimental data to a straight line using the method of least squares. The limits of detection (LOD) and quantification (LOQ) of the assay were evaluated as the concentrations of H_2O_2 giving CL signals corresponding to those of the blank plus three and ten times its standard deviation, respectively.

2.5.3. Quantification of intracellular H_2O_2 in human living cells

Standard solutions of the pro-oxidants menadione (3.2–50 μM), PMA (12.5–100 μM) and LPS (3.25–75 μg mL^{-1}) were prepared in PBS. HUVEC (10×10^3 cells $well^{-1}$), Caco-2 (25×10^3 cells $well^{-1}$) or HaCat (100×10^3 cells $well^{-1}$) cells were plated in the wells of a 96-well black microtiter plate for 24 h and then incubated for 20 min with 100 μL of the dioxetane CL probe working solution. Then, 100 μL of standard solutions of the pro-oxidant (PBS as the negative control) were added to induce intracellular H_2O_2 production and the CL emission was monitored for 40 min using a luminometric plate reader. The whole assay was conducted at 37 °C and the dose-response curve was obtained by plotting the CL signal versus the actual concentration of the pro-oxidant and fitting the experimental data to a straight line using the method of least squares.

2.5.4. Method validation

Method validation was performed by comparison with the commercial bioluminescent (BL) enzymatic assay (ROS-Glo™ H_2O_2 Assay from Promega, Madison, Wisconsin, USA) for the measurement of intracellular H_2O_2 after cell lysis. The BL emission was measured in black 96-well microtiter plates according to manufacturer's instructions using a luminometric plate reader.

2.5.5. Assay for antioxidant activity in human living cells

All the assays were conducted at 37 °C in 96-well black microtiter plates; CL signals were measured employing a luminometric plate reader.

Caco-2 cells were plated in the wells for 24 h at a seeding density of 25×10^3 cells $well^{-1}$ and then incubated for 20 min with 100 μL of PBS containing one antioxidant (Trolox, lipoic acid or gallic acid, concentration range 0.02–20 μM) and the dioxetane CL probe (10 μM). Then, 100 μL of PBS solutions of menadione (50 μM), PMA (100 μM) or LPS (75 μg mL^{-1}) were added and the CL emission was monitored for 40 min. The dioxetane CL probe working solution was used as the control.

B. juncea extracts were diluted with PBS to obtain concentrations ranging from 1.0 to 0.015 μg mL^{-1} (referred to the starting lyophilized material). Caco-2 cells were plated in the wells for 24 h at a seeding density of 25×10^3 cells $well^{-1}$and and treated for 24 h with 100 μL of the extracts. Upon removing the solution, cells were incubated for 20 min with 100 μL of the dioxetane CL probe working solution. Finally, 100 μL of PBS solution of menadione (50 μM) were added and the CL emission was monitored as described above.

2.5.6. Chemiluminescence imaging experiments

Imaging experiments were performed using an Olympus IX73 inverted microscope (Olympus Corporation, Tokyo, Japan) equipped with an ultrasensitive EM-CCD camera (ImagEM X2, Hamamatsu Photonics KK, Shizuoka, Japan) controlled by the proprietary software HCImage v. 4.2.6.1 and enclosed in a dark box to prevent interference from ambient light. Caco-2 cells (100×10^3 cells $well^{-1}$) were grown in a 24-well transparent microtiter plate for 24 h at 37 °C with 5% CO_2, then the medium was removed and the cells were incubated for 20 min at 37 °C with 500 μL of a 10 μM dioxetane CL probe working solution. Afterwards, 500 μL of a 50 μM solution of menadione was added and the CL emission was imaged for 30 min using a 5-min exposure time. Image processing was performed using the freely available Java-based software ImageJ v. 1.52d [34].

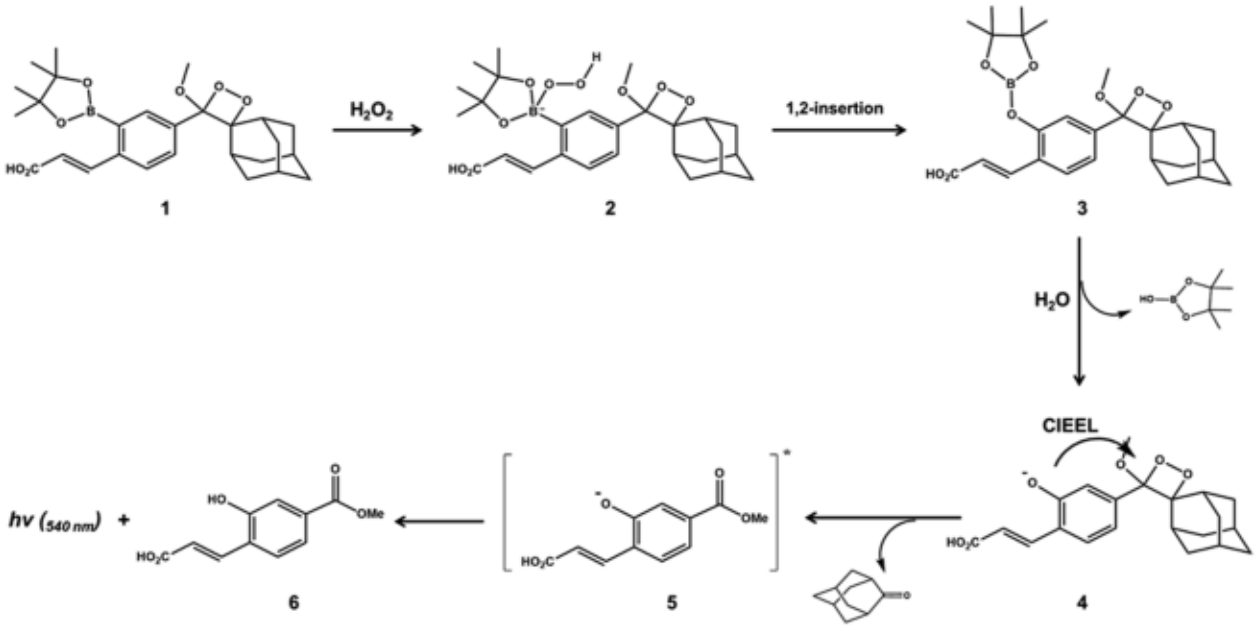

Fig. 1. Activation pathway of H_2O_2 CL probe, adapted with permission from Ref. [30]. Copyright (2017) American Chemical Society.

2.5.7. Data analysis

GraphPad Prism v. 6.05 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot the experimental data and for the least-squares fitting of calibration, dose-response curves (for evaluation of antioxidants and extracts IC_{50} values) and assay comparison graphs.

3. Results and discussion

3.1. Quantitative detection of H_2O_2 in cell-free system

First, the correlation between the CL emission and the concentration of H_2O_2 was investigated in a cell-free system. We were particularly interested in the possibility to detect small H_2O_2 amounts because we expected low intracellular H_2O_2 levels, which to our knowledge have never been quantitatively evaluated by CL.

As shown in Fig. 2A, CL measurements were carried out between 40 and 60 min after addition of the CL probe and the integrated CL emission in this time interval was used as the analytical signal. This signal integration period was chosen to avoid any contribution due to the nonspecific CL emission observed at short times, which is presumably owing to the presence of small amounts of free dioxetane in the probe (the CL probe itself is stable in the assay conditions: as shown in Ref. [30], RP-HPLC analysis indicated no decomposition even after 3 h at room temperature). The same signal integration period has been used in cell-based experiments, thus making the calibration curve suitable for evaluation of intracellular H_2O_2. The calibration curve showed a good linear correlation between the CL signal and the concentration of H_2O_2 up to 20 μM (Fig. 2B). The LOD and LOQ of the assay were 0.18 μM and 0.60 μM, respectively, which corresponded to 3.6×10^{-11} and 1.2×10^{-10} mol well^{-1} of H_2O_2.

3.2. Quantification of intracellular H_2O_2 in human living cells

The dioxetane CL probe was employed to measure the intracellular concentration of H_2O_2 in different human cell models upon pro-oxidant stimuli. We used a primary culture of human umbilical vein endothelial cells (HUVEC) and two immortalized cell lines, human colorectal adenocarcinoma cells (Caco-2) and human keratinocytes (HaCaT). Menadione, PMA and LPS were used as pro-oxidants. Menadione is a polycyclic aromatic ketone that increases intracellular H_2O_2 production by affecting mitochondrial respiration and glutathione depletion [35,36], PMA is known to stimulate intracellular NADPH oxidase [37] and LPS is a glycolipid component of the cell wall of Gram-negative bacteria commonly considered as a molecular pattern triggering the production of proinflammatory mediators and oxidative stress into cells [38].

Fig. 3A shows the kinetic profiles of the CL emission measured in Caco-2 cells upon treatment with different concentrations of menadione. As expected, the CL emission intensity increased with the concentration of the stimulant (Fig. 3B). By using the calibration curve

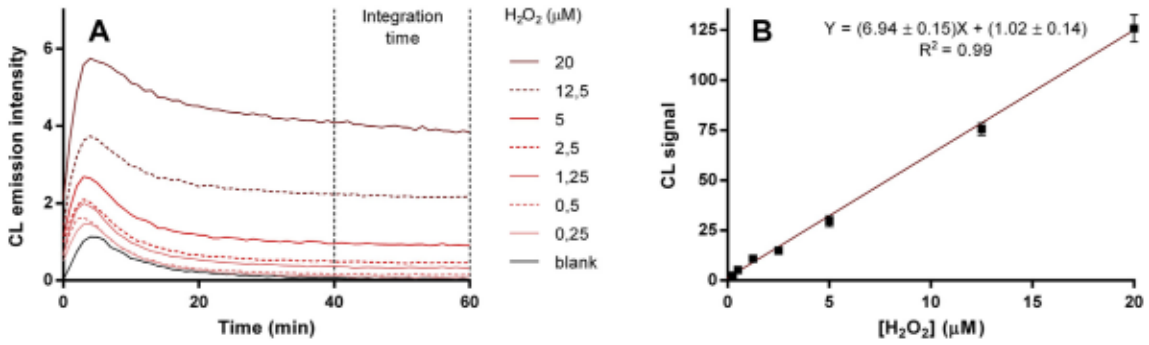

Fig. 2. (A) Chemiluminescence kinetic profiles obtained in PBS containing the dioxetane CL probe (5 μM) and different concentrations of H_2O_2. (B) Calibration curve showing the correlation between the CL signal and the concentration of H_2O_2. Each point represents the mean ± SD of three independent measurements.

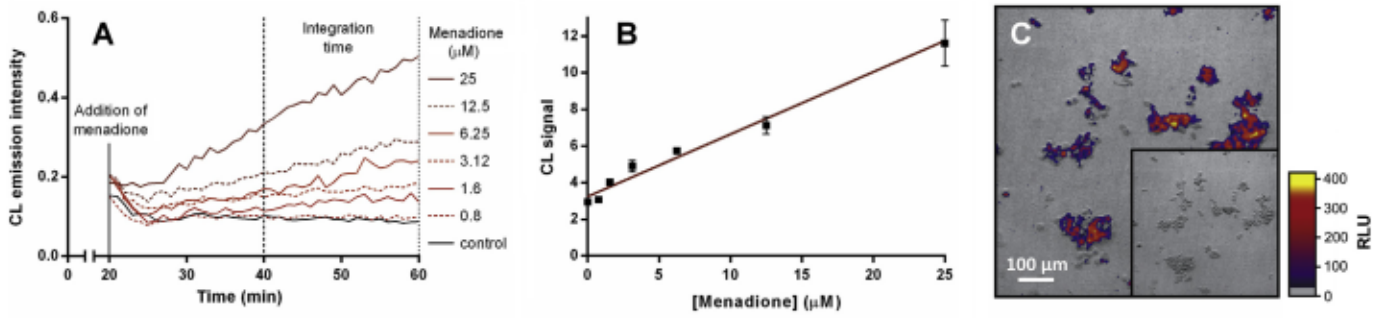

Fig. 3. (A) Chemiluminescence kinetic profiles obtained for Caco-2 cells in the presence of the dioxetane CL probe and different concentrations of menadione. (B) Dose-response showing the correlation between the CL signal and the concentration of menadione. Each point represents the mean ± SD of three independent measurements. (C) Overlay of CL image taken 20 min after addition of menadione (converted in pseudocolors according to the intensity of the emission) and of live image of Caco-2 cells (objective magnification 10X) obtained in the presence of the dioxetane CL probe and of 25 μM menadione (inset: live image of the cells). Bar represents 100 μm.

obtained in the cell-free system, the amount of H_2O_2 in Caco-2 cells treated with 25 μM menadione was estimated to be 1.6×10^{-10} mol well^{-1}, while the H_2O_2 content of control cells was comparable to the LOD of the assay, *i.e.*, around 3×10^{-11} mol well^{-1}.

Moreover, we evaluated the precision of our bioanalytical procedure, obtaining intra-assay CV% lower than 10.0% (< 25% Acceptance Criteria) and inter-assay CV% lower than 20.0% (< 25% Acceptance Criteria).

To confirm the intracellular localization of the emission of the probe, we also performed CL microscope imaging experiments on Caco-2 cells. The results (Fig. 3C) clearly showed that the CL emission is localized inside the cells, thus confirming the ability of the probe to penetrate in the cells and react with intracellular H_2O_2. This conclusion was also supported by the high value ($logD_{OW}$ = 5.49) of the octanol/water distribution coefficient of the probe evaluated in silico at pH = 7.4 using the AlogPs (v. 2.1) *on-line* tool [39].

To confirm the intracellular localization of the emission of the probe, we also performed CL microscope imaging experiments on the three cell models using all the pro-oxidant stimuli (menadione, LPS and PMA). Menadione-treated Caco-2 cells showed the highest CL signals (Fig. 3C), and the localization of the CL signals inside cells clearly demonstrated the ability of the probe to penetrate in the cells and react with intracellular H_2O_2. Moreover, we directly treated cells with H_2O_2 (10–100 μM) after medium replacement and we observed by microscope imaging experiments that the CL signal was mainly confined inside the cells, confirming the intracellular accumulation of the probe (data not shown). We have not routinely carried out the medium replacement to avoid cell detachment.

We also excluded the possibility that the CL probe is oxidized in medium and then the phenolate−dioxetane species diffuse into cells considering the higher cell membrane permeability of the boronate ester (LogP = 5.49 calculated by in silico analysis) compared to both phenol (LogP = 3.80) and phenolate (LogP = 3.54) intermediates. Moreover, the reaction of boronic ester with H_2O_2 is quite slow (k − 1–2 M−1s−1) [40], as also shown by the CL kinetics obtained in the cell-free system, thus the concentration of the CL probe in solution should remain high enough to effectively enter in cells.

A good correlation between the CL signal and the concentration of the pro-oxidant stimulus was observed for all the investigated cell models (Fig. 4 A-C and Table 1), which was consistent with the physiological regulation mechanisms of intracellular H_2O_2 production. The concentrations of pro-oxidants required to obtain a detectable increase in the CL signal were in the micromolar range for menadione and PMA (i.e., 1.5–3.0 μM for menadione and 2.0–10.0 μM for PMA, depending on the cell model) and ranged from 0.5 to 2.5 μg mL^{-1} for LPS. Interestingly, for all the pro-oxidants the highest CL signal increment was obtained in Caco-2 cells, which is consistent with their nature (Caco-2 cells originated from a colorectal adenocarcinoma and cancer cells are generally characterized by an increased H_2O_2 production rate and an impaired redox balance [41]). Since different cell models vary in both H_2O_2 production and, possibly, in permeability of cell membrane to the CL probe, we related the CL signal upon pro-oxidant stimuli treatment to the value of blank (i.e., untreated cells) in order to obtain just the increase of H_2O_2 level independently to the nature of cells.

Since a pro-oxidant stimulus causes cells to produce a variety of reactive species other than H_2O_2, probe selectivity represents a critical issue for the accurate quantification of H_2O_2. Green et al. [33] already investigated the selectivity of the H_2O_2 CL probe towards various ROS by measuring its response to *tert*-butyl hydroperoxide, hypochlorite, singlet oxygen, hydroxyl radical and *tert*-butoxy radical in a cell-free system. Peroxynitrite ($ONOO^-$) represents another potentially interfering species due to its rapid reaction with aromatic boronates at physiological pH [42]. Peroxynitrite derives from the reaction of superoxide with nitric oxide, which is mainly produced by enzymes belonging to the family of nitric oxide synthases (NOS), thus inhibition of NOS activity should efficiently suppress the production of $ONOO^-$ by cells. Therefore, to exclude any interference from $ONOO^-$ in the detection of H_2O_2 we also performed experiments in the presence of the cell-permeable competitive NOS inhibitor N(G)-monomethyl-L-arginine monoacetate (L-NMMA). As shown in Supplementary Fig. S1A, we didn't observe any significant CL signal change in Caco-2 cells treated with 25 μM PMA upon exposition to 100 μM L-NMMA for 6 and 24 h. We also investigated the CL signals in cells preincubated with 50 ng/mL of TNFα for 24 h, able to induce the expression of the inducible NOS isoform (iNOS) [43]. Supplementary Fig. S1B clearly showed that even in this case a contribution to the CL emission due to the reaction of the probe with $ONOO^-$ can be excluded. These results suggest that the bioassay could be used to selectively quantify H_2O_2 in real time in living cells. According to the literature, at physiological pH the reaction between $ONOO^-$ and boronates is very fast [42]. Therefore, the absence of any significant contribution to the CL signal due to the reaction of the probe with $ONOO^-$ can be explained assuming that intracellular peroxynitrite levels are lower than that of H_2O_2. It should be also considered that the higher specificity of $ONOO^-$ towards aryl-boronate esters was mainly tested using concentrations hundreds of folds higher than the physiological levels [44].

3.3. Method validation

Being a cellular standard reference material with known intracellular H_2O_2 content not available, the assay accuracy was evaluated by comparing our results with those obtained employing ROS-Glo™ H_2O_2 assay, a BI. enzymatic assay that measures H_2O_2 on lysed cells. This assay employs a modified luciferin substrate that, upon cell

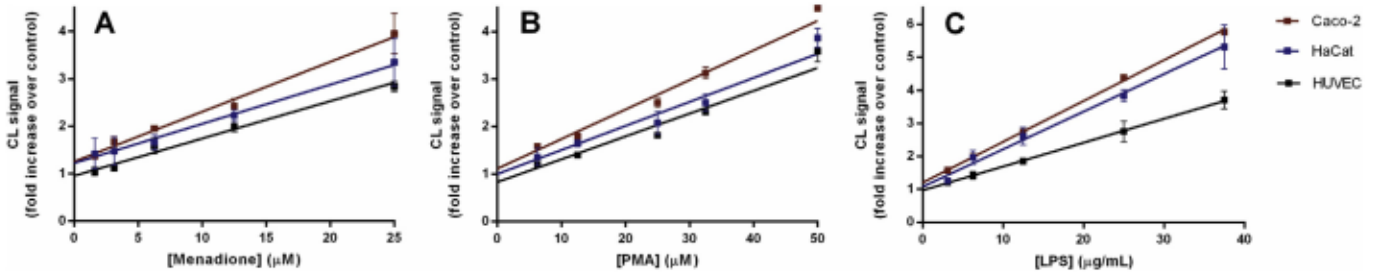

Fig. 4. Chemiluminescence signals obtained in Caco-2, HaCat and HUVEC cells in the presence of the dioxetane CL probe and of different concentrations of (A) menadione, (B) PMA, and (C) LPS. To facilitate the comparison between different inductors or cell types, the CL signals were reported as the ratio between the signal obtained in the presence of the pro-oxidant and the signal of the control. Each point represents the mean ± SD of three independent measurements.

lysis, reacts with H_2O_2 generating a luciferin precursor, which is then converted to luciferin upon addition of a solution containing D-cysteine. Finally, luciferin is detected by BL upon addition of a recombinant luciferase. Even if this method used cell lysates and requires longer assay times, results are comparable. For all cell types, we found a good correlation between the luminescence signals obtained in the presence of increasing concentrations of menadione (Fig. 5), thus demonstrating the ability of the dioxetane CL probe to provide accurate results. Furthermore, in comparison to the ROS-Glo™ Assay, the CL probe allowed easier and real-time measurement of intracellular H_2O_2 even in a small quantity of cells, avoiding long reagent preparations (the CL probe is the only reactant) and time-consuming pre-treatment procedures (no cell lysis is required).

3.4. Cell-based assay for antioxidant activity

Finally, we used the dioxetane CL probe to develop a cellular CL assay for measuring antioxidant activity. As standard antioxidant compounds we used well-known molecules such as Trolox (a water-soluble analogue of vitamin E), lipoic and gallic acids. Trolox has advantages over α-tocopherol, which is lipid soluble, because it can be enter in both aqueous and lipidic compartments of cells [45]. Moreover, Satoh et al. have claimed that the antioxidant property of Trolox surpasses that of α-tocopherol [46]. α-Lipoic acid itself is not a strong antioxidant, but its reduction within cells produces the dithiol derivative (dihydrolipoic acid, DHLA), a more powerful antioxidant [47]. PEG-conjugated catalase was not assayed as an intracellular antioxidant because of its complex and quite long mechanism of access into the cell [48,49].

Simultaneous treatment of cells with pro-oxidants and various concentrations of antioxidants in the presence of the dioxetane CL probe allowed to measure the decrease of intracellular H_2O_2 due to the presence of antioxidants, then to obtain the dose-response curves (data not shown) and the antioxidant IC_{50} values. Table 2 shows the IC_{50} values of Trolox and lipoic acid obtained in Caco-2 cells using different pro-oxidant stimuli. No results are shown for gallic acid because the CL signal increased with the concentration of the antioxidant (data not shown). We attributed this behaviour to the generation of H_2O_2 in solution due to the reaction of gallic acid with atmospheric oxygen, a reaction already reported for various phenolic compounds [50,51].

No cytotoxic effect (assessed by LDH release in the cell medium) was observed in treated cells, thus excluding the possibility that the decrease in H_2O_2 concentration was due to nonspecific effects on cell metabolism during the experiments (data not shown).

To confirm the ability of the bioassay to identify new physiological antioxidants, we measured the antioxidant activity of extracts derived from different tissues of *B. juncea* (oriental mustard), which contains the characteristic glucosinolate-myrosinase endogenous plant defence system of the *Brassicaceae* family. Its products (mainly isothiocyanates) have a recognized antioxidant activity, being powerful inducers of mammalian cytoprotective proteins through the Keap1–Nrf2–ARE pathway (the major regulator of cytoprotective responses to oxidative and electrophilic stress) [52].

According to the cell-based assay, the IC_{50} values of the extracts were in the order of 0.1–0.2 μg mL^{-1} (referred to the starting freeze-dried powders). To our knowledge, the high content of ITCs (supplementary materials), especially in *B. juncea* roots, can explain the reduced response to pro-oxidant stimuli observed in Caco-2 cells after a 24 h incubation with the extracts. Leaves and stems extracts, despite their lower content of ITCs compared to roots, gave similar IC_{50} values, probably because of other antioxidant molecules with a synergic effect with ITCs, as already described in literature for sulforaphane [53,54].

As in previous experiments, monitoring of LDH release in cell medium excluded any cytotoxic effect of the extracts (data not shown).

4. Conclusions

One of the challenges in studying ROS in complex matrix is robust detection, since ROS are short-lived and their direct detection is seldom feasible. Intracellular H_2O_2 level can reasonably reflect a general change in the intracellular ROS production when various ROS are converted to H_2O_2 within cells [3], therefore the selective measurement of H_2O_2 over different ROS is critical for the accurate evaluation of oxidative stress.

Here, we report the development and optimization of a ~~rapid and~~ selective intracellular antioxidant bioassay based on an adamantylidene - 1,2 - dioxetane probe containing an arylboronate moiety. The probe, in the presence of H_2O_2, is converted to the correspondent phenol, which emits green light with high efficiency. The assay low LOD enables the quantitative detection of H_2O_2 in complex biological samples,

Table 1
Slopes and coefficients of correlation (R^2) of the linear relationships between the CL signal and the concentration of pro-oxidant stimulus obtained in Caco-2, HaCat and HUVEC cells. Each result is the mean ± SD of the independent experiments.

	Caco-2 cells		HaCat cells		HUVEC cells	
Pro-oxidant stimulus	Slope ± SD	R^2	Slope ± SD	R^2	Slope ± SD	R^2
Menadione	0.105 ± 0.007	0.988	0.083 ± 0.005	0.991	0.079 ± 0.007	0.977
PMA	0.062 ± 0.006	0.977	0.051 ± 0.006	0.963	0.048 ± 0.006	0.956
LPS	0.124 ± 0.002	0.999	0.114 ± 0.008	0.985	0.072 ± 0.001	0.999

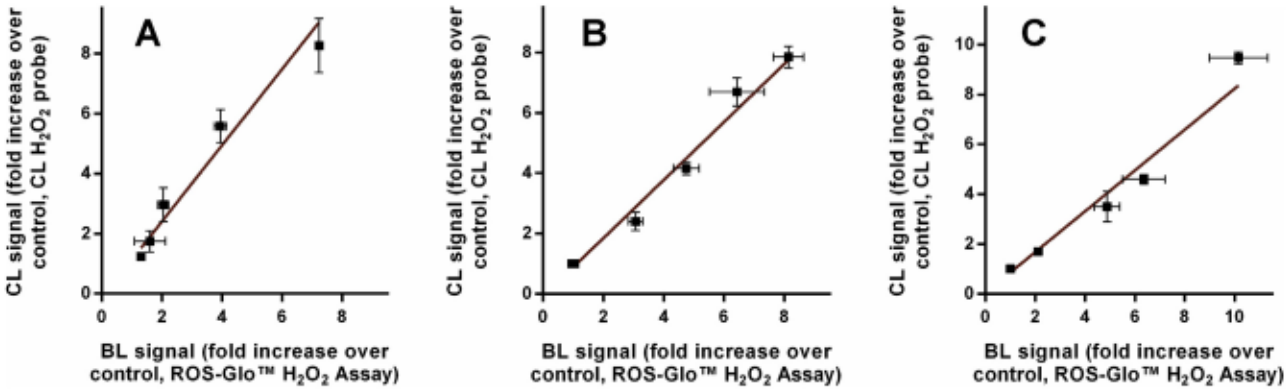

Fig. 5. Comparison of the luminescence signal observed in (A) Caco-2, (B) HaCat, and (C) HUVEC cells in the presence of different concentrations of menadione (up to 50 μM) using the CL H_2O_2 probe and the ROS-Glo™ H_2O_2 Assay. To facilitate the comparison between the assays, the signals were reported as the ratio between the signal obtained in the presence of the pro-oxidant and the signal of the control. Each point represents the mean ± SD of three independent measurements.

Table 2
IC_{50} values measured in Caco-2 cells for the reference antioxidants Trolox and lipoic acid using different pro-oxidant stimuli. Each value represents the mean ± SD of three replicate measurements.

Pro-oxidant	IC_{50} (μM)	
	Trolox	Lipoic acid
Menadione[a]	0.23 ± 0.07	2.05 ± 0.18
PMA[b]	0.68 ± 0.03	1.52 ± 0.02
LPS[c]	0.19 ± 0.05	0.51 ± 0.03

[a] Concentration 25 μM.
[b] Concentration 50 μM.
[c] Concentration 37.5 μg mL^{-1}.

such as human living cells in physiological conditions. Notably, even small changes in intracellular H_2O_2 concentration in the presence of oxidants and antioxidants could be evaluated, which could provide a rapid first-level antioxidant screening in complex biological matrices for pharmaceutical and nutraceutical field.

The developed method is characterized by a good precision and is able to produce relative information that quantitatively scores an increased or decreased of intracellular H_2O_2 production with respect to basal level, as a consequence of pro- or antioxidant treatment.

These results were achieved with, as far as we know, the shortest incubation time ever used for a luminescence-based bioassay. Moreover, our cell-based bioassay is one of the fastest assays among other intracellular H_2O_2 selective CL bioanalytical methods [55–63].

For the first time, scientists now have an effective, rapid, reproducible, robust single-entity CL tool that can be used to evaluate biological processes, i.e. intracellular H_2O_2 production. This rapid cell-based bioassay has the potential to provide both the researcher and the diagnostician with a powerful tool to look within the cells, in real-time, at the fundamental biochemistry and production of H_2O_2 and correspondingly the redox state of the metabolism.

CRediT authorship contribution statement

D. Calabria: Investigation, Formal analysis, Methodology. **M. Guardigli:** Formal analysis, Writing - review & editing, Software, Supervision. **M. Mirasoli:** Writing - review & editing, Funding acquisition. **A. Punzo:** Investigation, Formal analysis. **E. Porru:** Visualization, Validation. **M. Zangheri:** Visualization, Validation. **P. Simoni:** Visualization, Validation, Resources. **E. Pagnotta:** Investigation, Formal analysis, Data curation. **L. Ugolini:** Data curation. **L. Lazzeri:** Visualization, Validation. **C. Caliceti:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing - original draft. **A. Roda:** Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2020.113760.

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